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**(54) Title of the Invention : A Method of Analysis by Use of a Bio -
Functional Material Fixation Electrode**

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Specification

1. Title of the Invention : A Method of Analysis by Use of a Bio -
Functional Material Fixation Electrode

2. What we claim is

A method of analysis in a system by which detection of, or determination
of a concentration of, a material to be measured is made by an electro -
chemical non- steady method by use of an electrode to which a bio -
functional material is fixed;

applications for patents (Showa 62 / 1987 - Patent Application No. 55387, and - Patent Application No. 56472). In addition, the inventors of the present invention have developed an analysis system which utilizes such a fixed electrode and is capable of making a measurement of a minute amount of a sample which remains still, and a method of analysis based on an electro - chemical non - steady method, which utilizes the above - mentioned, and filed for a patent (Showa 62 / 1987 Patent Application No. 304523).

This method has expanded the feasibility of an application scope of a bio - sensor in that it is characterized in that it does not require to strictly define the amount to be collected of a sample solution, is capable of making a measurement of a minute amount of a sample at still and a non - dilution measurement, and makes it possible to make a rapid measurement of an order of milli - seconds. However, it is necessary to make, in advance, a blank measurement of a buffer solution (hereinafter referred to as blank solution) which does not contain a material to be measured, and to deduct it from a response for a target sample solution,

and this operation becomes complicated. Because of this problem, although this method is a rapid analysis method which utilizes a response of an order to milli seconds, it fails to fully utilize the advantages thereof as a whole measurement operations including the operation prior to the measurement of a sample solution.

[Problems which the Invention Tries to Solve]

When a non - steady response current with respect to a constant potential simple pulse is recorded by applying an electro- chemical non - steady method to an enzyme electrode prepared by embedding and fixing an enzyme onto a platinum black electrode, the response current thereof does not become zero with respect to the blank solution as shown in Example 2. This non - steady current contains a Faraday current derived from an electro - chemical oxidation reduction reaction on the electrode surface and impurity molecules in addition to a charging current of the electric double layer capacity immediately after the application of a pulse. Because of this, it is necessary to deduct the non - steady current value observed for the blank solution from the non - steady current value

observed for the sample to be measured. In addition, in a case in which the response for this blank solution is not sufficiently small to be neglected in comparison with the response current for the sample to be detected, the reproducibility and reliability of the sensor response are damaged. Therefore, the present invention is to provide a method of analysis which does not require the measurement of a response with respect to a blank solution and the so-called blank measurement, and by this, a rapid analysis method with good reproducibility and excellent reliability is to be provided.

[Constitution of the Invention]

[Means by which to Solve these Problems and the Actions]

In order to solve the problem points described above, the present invention is to provide a method of analysis characterized in that a preliminary pulse is applied immediately prior to the application of a measurement pulse, any response which is not derived from a material to be measured, by an electro-chemical preliminary treatment, and then after maintaining an open circuit state for a certain period of time, a

sensor response is measured by a subsequent measurement pulse.

The electrode in and to which a bio - functional material to be utilized in the present invention is embedded and fixed and the system of analysis are the system of analysis which utilizes a non - steady response described in the patent application (Showa 62 / 1987 Patent Application No. 304523). That is to say, as described in Showa 62 / 1987 Patent Application No. 55387 and Patent Application No. 56472, it is an electro- chemical system which has 3 electrodes, that is, an electrode of a structure having an electrically conductive micro particle layer obtained by embedding and fixing a bio - functional material such as an enzyme onto the surface of a minute electrode constituted with micro particles of platinum, etc. which is used as an active electrode, a reference electrode made of silver - silver chloride, etc., and an opposing electrode, and one example having such a structure is shown in Fig. 1. In Fig. 1, an active electrode 1 is a micro electrode onto which a bio - functional material (for example, glucose oxidizing enzyme) is embedded and fixed, and it is

a micro electrode of a diameter, for example, in a range of about 1 μm to 500 μm . A sensor 6 is constituted with an opposing electrode 2 made of a platinum wire and a reference electrode of a silver / silver chloride series as well as the above - mentioned electrode. The above - mentioned 3 electrodes, that is to say, the fixed micro electrode 1, the opposing electrode 2 and the reference electrode 3, are embedded with a polyethylene resin 4 in a hole of a frame 5 made of Teflon. Since such a sensor element 6 has a structure in which 3 thin metal wires are embedded and fixed, it is possible to make the whole sensor as an extremely minute sensor if use is made of a fine processing technique.

If use is made of this sensor element, it is possible, for example, to make a measurement of a minute sample of an amount of about 1 μl . That is to say, by a method in which a potential is applied after a minute amount of a sample is dropped in, and then a current value generated at that time is detected, it becomes possible to detect a material in a minute amount of a sample.

A sensor response is obtained by recording a non - steady current

response to a constant potential pulse by utilizing the above - mentioned analysis system, however, in a case of a response to a simple pulse, even with respect to a solution which does not contain a material to be measured, a Faraday current of a non - negligible degree is detected as shown in Example 2. The reason why it has been necessary to make a blank measurement before is that it is necessary to deduct this current. As shown in Fig. 2, with the present invention, immediately prior to the application of a constant potential pulse 9 for the measurement, a preliminary constant potential pulse 7 is applied, and after maintaining an open circuit state for a certain period of time, a pulse 9 for measurement is applied, thereby resolving the above - mentioned problem point.

[Action]

The non - steady current response observed at the time when a constant potential pulse is applied to an electro- chemical system, is in general constituted with a capacity current derived from the charging of an electric double layer capacity and a Faraday current derived from an electro - chemical oxidation / reduction reaction at an electrode. As the

capacity current attenuates with a time constant of several ten micro sec to several hundred micro second, it is permissible to consider that it is only the Faraday current that matters for a non - steady current which is of an order of several milli sec to several ten milli sec.

It may be considered that a Faraday current for a blank solution is mainly due to an electrode reaction of impurities contained in a solution and an electro - chemical oxidation / reduction reaction on the surface of an electrode. In the following explanation and examples embodying the invention, we shall discuss a case in which it is applied to the detection of glucose. In this case, glucose is oxidized by the action of a glucose oxidizing enzyme fixed to the surface of the electrode, and it is devised to detect glucose by detecting a current required to electro - chemically oxidize hydrogen peroxide generated this time. Therefore, in the following description, for both preliminary pulse and measurement pulse, oxidizing pulses are utilized, however, depending on a combination of a bio - functional material and a material for fixing, there may be case in which a reduction pulse is to be applied.

There is a possibility that an oxidation current observed when a constant potential pulse of 0.5 V is applied to a silver - silver chloride reference electrode, may include, in addition to the oxidation current of hydrogen peroxide, an oxidation current of reducing impurities in the neighborhood of the electrode, and an oxidation current generated at the time when a surface oxide / oxides is / are generated through the oxidation of the platinum black surface. It is considered that a non - steady current observed for a blank solution is mainly derived from an oxidation current of reducing impurities and an oxidation current of a platinum black surface. Therefore, since the application of a preliminary pulse prior to a measurement pulse produces an effect to oxidize reducing impurities which are present in the neighborhood of the surface of the electrode and to adjust the oxidized state of the platinum black surface to a state similar to a state obtained in a case in which a pulse for measurement is applied, with respect to a blank solution, it becomes possible to make it small to the extent to be able to neglect a response of a blank solution.

With respect to a sample solution which contains glucose, hydrogen

peroxide which has already been present is oxidized similarly by a preliminary pulse, and since hydrogen peroxide is again given off by a reaction of glucose during the period in which the open circuit state of 8 is maintained, it becomes that hydrogen peroxide generated at this time is detected by a measurement pulse 9.

[Examples embodying the Invention]

Example 1 Preparation of a sensor element

The sensor shown in Fig. 1 was prepared by the procedures described below. A minute platinum wire of a diameter range of 1 μm to 500 μm , a platinum wire for an opposing electrode of 200 μm in diameter, and a silver wire of 500 μm to 1 mm in diameter were sealed in one each in a Teflon frame 5 with a polyester resin 4, it was polished with an alumina polishing agent. An enzyme was fixed to the surface of the platinum active electrode by the following method.

Platinum black was electrolytically deposited by the constant potential electrolysis for 5 minutes at a potential of -0.1 V with respect to the silver - silver chloride reference electrode in a 3 % platinum chloride fusing

agent liquor which contained lead acetate of 300 ppm thereby obtaining platinum black of about several μm in thickness. Next, after the platinum black deposition electrode thus obtained was dried at room temperature for 60 seconds, it was maintained in 0.5 M sulfuric acid aqueous solution at -0.3 V for 30 minutes, and thus hydrogen was allowed to be given off from the platinum black electrode. After drying it in wind for 60 sec., a constant potential of 1.2 V was applied thereto for 15 minutes, thereby performing an oxidation treatment of the electrode surface, it was immersed in 1 ml of a phosphoric acid buffer solution containing glucose oxidizing enzyme of 5500 units (ph 6.5) for 30 minutes, followed by drying in wind again.

Next, in the sensor element 6 having a micro electrode obtained as described above, a silver wire was used as a silver - silver chloride reference electrode. The sensor element 6 consisting of the 3 electrodes made in this manner was subjected to agitation in 0.1 phosphoric acid buffer solution in one day and night, and thus the sensor element having 3 electrodes to be used in the present invention was obtained.

Example 2 Measurement of the concentration of glucose by use of a simple pulse

In measuring, a concentration of glucose, use was made of a measuring system shown in Fig. 3. That is to say, the active electrode 1, the opposing electrode 2 and the reference electrode 3 of the sensor element 6 were connected respectively to the potentiostat 10, and for the application of a pulse, the potentiostat was driven by a signal from the function generator 11. The non-steady current was recorded by the digital memory scope 12. The fixation enzyme electrode prepared by use of a platinum wire of 50 μm in diameter was used as an active electrode, and the non-steady current response obtained at the time when a simple constant potential pulse of 0.6 V was applied to the silver-silver chloride electrode is shown in Fig. 4. The curve 13 is a response to the phosphoric acid buffer solution which contained 20 mM glucose, 14 is a response to a blank solution which contained only the phosphoric acid buffer solution, and 15 is a response to a phosphoric acid buffer which contained 20 mM fructose.

It is considered that in any of the responses, the initial peak current which seems to be due to the charging of the double layer capacity attenuates in about 1 milli second, and that after 2 seconds, the Faraday current is observed. Since the fructose solution gives a curve almost similar to the curve of the blank solution, it is considered that the difference between the curve 13 and the curve 14 is derived from the current flowing when hydrogen peroxide generated by the presence of glucose of about 20 mM is electro - chemically oxidized. Therefore, the difference in current responses of the glucose solution and the blank solution after 2 milli seconds is defined as a sensor response, and the sensor responses at various concentrations were measured. The results of this measurement are shown in Fig. 5, and the sensor responses renders one - to one correspondence to the concentrations, and thus it is possible to know the glucose concentrations from the responses. However, in order to obtain the data shown in Fig. 4 with good reproducibility, it was necessary to maintain the time from the contact between the sensor and the solution to the start of measurement constant, and in addition, it was necessary to

measure the response to a blank solution each time.

Example 3 Measurement of a glucose concentration by use of a preliminary pulse

By using the same equipment as those used in Example 2, and after giving the preliminary pulse 7 shown in Fig. 2, the open circuit state 8 was maintained for a certain period of time, and thereafter, a measurement by use of a measurement pulse 9 was carried out. After a constant potential pulse of 0.6 V was given to the silver - silver chloride reference electrode for 60 seconds, an open circuit state was maintained for 10 seconds, and following this, a constant potential measurement pulse of 0.6 V was applied, thereby obtaining a non - steady current response as shown in Fig. 6. The curve 16 is a response to 5 mM glucose solution and the curve 17 is a response to the blank solution.

The response to the blank solution was about 1.5 μ A at about 5 milli seconds, and after 10 milli seconds, it became small enough to be neglected in comparison with the response current to the glucose solution. That is to say, in a case in which use is made of a preliminary

pulse, as the response of the blank solution is extremely small, it is not necessary to do the operation to measure the response to the blank solution each time and to make a deduction of that current value.

Example 4 Glucose concentration dependency of a sensor response

In order to determine a glucose concentration from the non - steady current response obtained in Example 3, the relation between the current value and the glucose concentration at a certain time period after the application of a measurement pulse.

After a preliminary pulse was applied to sample solutions of various glucose concentrations for 20 seconds similarly to Example 3, an open circuit state was maintained for 10 seconds, following this a non - steady current with respect to the constant potential measurement pulse was measured, and with the current value after 10 milli seconds as a sensor response, the glucose concentration dependency was measured. The results are shown in Fig. 7. In the glucose concentration range from 0.1 M to 10 mM, good linearity is shown, and this indicates that a glucose concentration can be directly determined from the non- steady current

value with respect to the measurement pulse without making any measurement of a response to a blank solution by use of a preliminary pulse.

Example 5 Pulse application time and sensor response

By using the same equipment as those used in Example 2, and under the same potential conditions as those in Example 3 and Example 4, the relation between the pulse application time and the sensor response in Fig. 2 was studied. By varying the preliminary pulse application time from 1 second to 60 seconds, and the open circuit state was maintained for 10 seconds, and then the non - steady current values were measured after 10 milli seconds. The results are shown in Fig. 8. The curve 18 shows the response to the phosphoric acid buffer solution containing 5 mM glucose, and the curve 19 shows the response to the blank solution. The response to the blank solution rapidly decreases up to about 15 seconds of the preliminary pulse and it takes about 60 seconds for it to sufficiently attenuate. On the other hand, the response to glucose assumes approximately a constant value at around 30 seconds.

On the other hand, in order to study the relation between the open circuit time and the sensor response, the preliminary pulse time was kept at 20 seconds, the open circuit time was changed from 1 second to 30 seconds, and the sensor response defined at the non - steady current value of about 10 milli seconds was studied. The results are shown in Fig. 9. The curves 20, 21 and 22 are respectively response for glucose of 10 mM, 5 mM and 2 mM. From this result, it can be considered that in the open circuit state, the oxidizing reaction of glucose mainly due to the glucose oxidizing enzyme proceeds and the generation of hydrogen peroxide progresses on the surface of the electrode.

If we summarize the above- mentioned results, it may be seen that the longer the preliminary pulse time, the smaller becomes the response given by the blank solution and that the longer the open circuit time, the larger becomes the sensor response. On the other hand, from the practical view point, the shorter these times, the more advantageous for the rapid analysis. Therefore, under the condition that the preliminary pulse was 5 seconds and the open circuit time was 5 seconds, the sensor

responses for glucose solutions of various concentrations were measured.

The results are shown in Fig. 10.

The response for a case in which the glucose concentration is zero, that is, for the blank solution, does not become zero, however, good sensor responses with good reproducibility have been obtained for each concentration of glucose, and if a preliminary pulse is given, there are cases in which measurements with good reproducibility become possible even if one does not wait until the response becomes completely zero for the blank. Thus, it is suggested that it is possible to make such a measurement rapid under this condition, as required.

Example 6 Study of the reproducibility of responses

In order to study the improvements of the reproducibility by the application of a preliminary pulse, the sensor responses were repeatedly measured both for a case in which a preliminary pulse was used and for a case in which a simple pulse was used. The measuring equipment was the same as the one used in Example 2, and with 10 mM glucose solution as a sample, the preliminary pulse time was 20 seconds and the open

circuit time was 30 seconds. One example of the results is shown in Fig. 11.

The white circles in Fig. 11 are the sensor responses when the preliminary pulse was not used, and only the simple pulse was used, and the black circles give the results when the above - mentioned preliminary pulse was used.

In the case of the simple pulse, the first response always gives an abnormally large value, and with respect to the second and subsequent responses, a scatter of about 3 % in terms of the standard deviation was always observed. On the other hand, in the case in which the preliminary pulse was used, the reproducibility was improved and the standard deviation became about 0.4 %.

[Effects of the Invention]

The application of the electro- chemical non - steady method to an enzyme embedded electrode for use in detection of a bio - substance has open a road for the establishment of a rapid analysis method which does not depend on the quantity of a sample, however, there have been

problems in terms of conveniences as it requires a blank measurement, etc., and thus it has been practically difficult to utilize it for the self control, etc. of a blood sugar value of a patient suffering from diabetic retinopathy, etc. as is. By the combined use of a preliminary pulse and an open circuit state in accordance with the present invention, as shown in Example 3, Example 4 and Example 5, it has become possible to make the operations markedly simple and easy, and furthermore as shown in Example 6, the reliability has been markedly improved.

That is to say, as described above, the technical effects of the present invention lie in the fact that for a minute amount of a sample, a simple highly sensitive rapid analysis with high reliability has become possible.

4. Simple Explanation of the Drawings

Fig. 1 is an example of the constitution of a sensor element which is to be used in the present invention.

Fig. 2 shows one example of a program of a preliminary pulse and an open circuit state to be used in the present invention.

Fig. 3 is a conceptual diagram of a measurement system used in Example 2 through Example 6.

Fig. 4 is an example of a non - steady current response when a constant potential simple pulse is applied.

Fig. 5 shows the dependency of a sensor response on a glucose concentration obtained by use of a non- steady current response for a constant potential simple pulse.

Fig. 6 is an example of a non- steady current response when use is made of a preliminary pulse.

Fig. 7 shows a glucose concentration dependency of a sensor response obtained from a non - steady current response when a preliminary pulse was used.

Fig. 8 shows the dependency of a sensor response on the preliminary pulse time.

Fig. 9 shows the dependency of the sensor response on the time duration of the open circuit state.

Fig. 10 shows the sensor responses when both the preliminary pulse and

the open circuit state are set to 5 seconds.

Fig. 11 compares the reproducibility of the sensor responses obtained from the responses to a simple constant potential pulse and the sensor responses obtained in a case in which a preliminary pulse is used.

[Explanation of the Symbols of the Major Components or Parts]

1 is a bio - functional material fixed electrode, 2 is an opposing electrode, 3 is a silver - silver chloride reference electrode, 4 is a polyester resin resin (sic), 5 is a Teflon type frame, 6 is a sensor element, 7 is a preliminary pulse, 8 is an open circuit period, 9 is a constant potential measurement pulse, 10 is a potentiostat, 11 is a function generator, 12 is a digital memory scope, 13 is a response to 20 mM glucose, 14 is a response to a blank solution, 15 is a response to a 20 mM fructose solution, 16 is a response to a 5 mM glucose, 17 is a response to a blank solution, 18 is a response to a 5 mM glucose, 19 is a response to a blank solution, 20 is a response to a 10 mM glucose, 21 is a response to a 5 mM glucose, and 22 is a response to a 2 mM glucose.

Applicant : National Disabled Persons Rehabilitation Center

Fig. 1

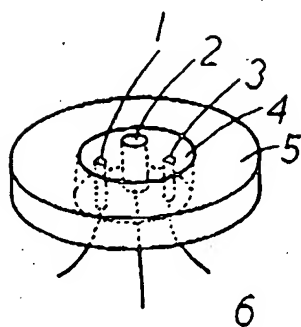


Fig. 2

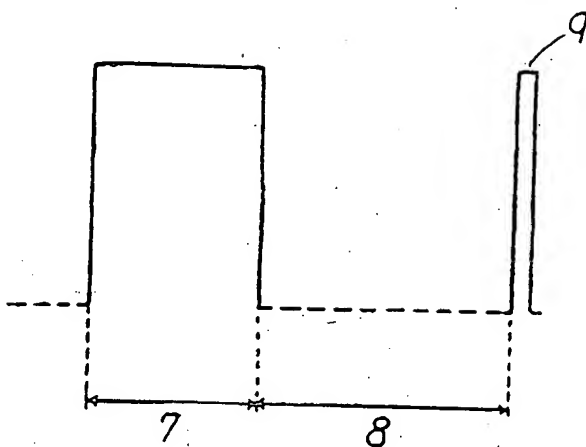


Fig. 3

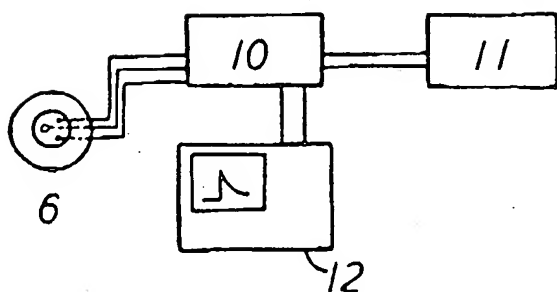
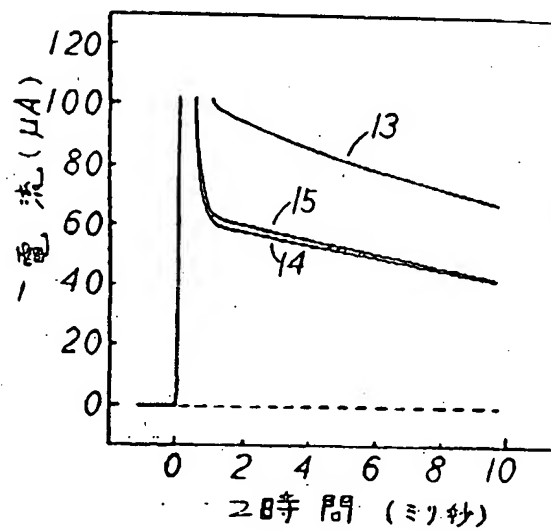


Fig. 4



key 1 current, 2 time (milli sec)

Fig. 10

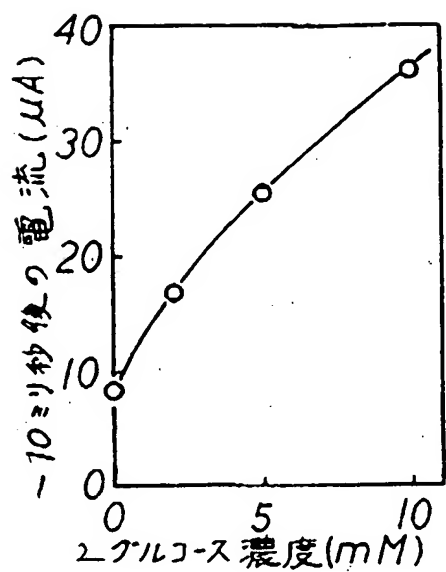
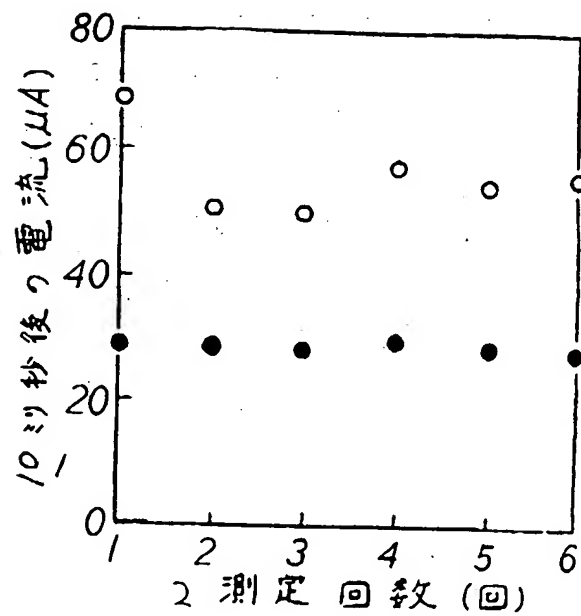


Fig. 11



key 1 current after 10 milli sec,

2 glucose concentration

Key 1 current after 10 milli sec

2 No. of measurements